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Protein Components of Bacteriophages λ and λ Virulent

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Parallel studies have been made of the protein coats of the temperate bacteriophage λ and of a deletion mutant, λ virulent. A new method for preparing ghosts of both phages by the action of Cu^{++} is described. Protein ghosts of both phages can be dissolved in citrate at *p*H values below 3, more rapidly in the presence of 8 M urea. Both phages yielded three apparently identical protein components which can be separated by thin-layer gel filtration and thin-layer gel electrophoresis. The protein of molecular weight 47,000 ± 1,500 represents about 55% of the protein of the ghosts and is therefore likely to be the subunit of the head. The other proteins of molecular weight 30,000 ± 1,500 and 16,000 ± 1,500 represent approximately 25% and 20% of the protein, respectively. Amino acid analyses of the ghosts from the two phages have been carried out and show no significant differences. The buoyant density of phage λ virulent is 0.016 g/ml less than that of λ. Since no differences have been found in the protein components of the two phages, this indicates that the virulent mutant contains approximately 16% less deoxyribonucleic acid than the temperate phage.

Temperate bacteriophage λ has been the object of much genetic study. Relatively little is known however about the anatomy of phage λ and its mechanism of infection. Electron microscopic examination of λ reveals only two structures: a head, which may be hexagonal in cross section, and a long thin tail with no accessory structures (10). To study the structure in more detail, an attempt has been made to dissociate the virus in a step-by-step fashion, to isolate its parts, and to study the chemical structure of the protein coat. A parallel study of structure has been undertaken for a virulent mutant of λ (hereafter referred to as λ v) to determine whether structural differences exist which can be associated with virulence.

MATERIALS AND METHODS

Preparation and purification of phage stocks. The preparation and purification of large quantities of phage λ was achieved by a modification of earlier procedures (10). *Escherichia coli* W-3104/λ was grown at 37 C to a density of 0.8×10^9 to 1×10^9 bacteria per ml in Fraser medium (6) in which the $MgSO_4$ concentration had been increased to 10^{-2} M. The culture, in 8-liter batches, was passed through a modified ultraviolet induction apparatus (designed and constructed by R. P. Mackal) in which the bacterial suspension was forced through a spiral quartz tube with an inside diameter of 6 mm by a stainless-steel pump at a rate of 1 to 1.2 liters per min. Irradiated cultures were incubated at 37 C with vigorous aeration. Lysis was complete in 2.5 to 3 hr. The crude lysate containing about 4×10^{10} to 8×10^{10} λ phages per ml was concentrated in the cold to one-third the original volume by immersion of cellulose casing filled with Carbowax 6000. The concentrated lysate was clarified by centrifuging at $6,000 \times g$ for 15 min in an angle centrifuge. The phages were then sedimented in the International model BD-2 at $57,000 \times g$ for 150 min. The pooled pellets were washed once in a tris(hydroxymethyl)aminomethane(Tris) buffer containing 0.067 M Tris, 0.5% NaCl, and 0.025% $MgSO_4$ at *p*H 7 and sedimented. The phage pellets were further purified by CsCl density gradient centrifugation at 37,000 rev/min in the Tris buffer containing 700 mg of CsCl per ml in the Spinco SW-39 rotor for 18 hr at 3 C. The phage appeared as a white layer in the center of the tube. The phage samples were dialyzed against the Tris buffer to remove CsCl. A second CsCl gradient purification was used to remove traces of cell debris and impurities. An amount of 5 to 6 ml of 2×10^{13} phages per ml was recovered. This corresponds to 50% recovery.

Phage λ v was prepared from *E. coli* W-1485/T1 by virulent replication. *E. coli* W-1485/T1 was grown at 37 C in Fraser medium in 8-liter batches to a cell density of 6×10^8 to 7×10^8 bacteria per ml. Infection with λ v was carried out at a multiplicity of 2 to 3 phages per bacterium. The infected cultures were grown with aeration for 5 hr. The crude lysate contained 0.6×10^{11} to 1×10^{11} phages per ml.

The purification of phage λ v was effected by the same procedure used for phage λ with the exception of the CsCl density gradient centrifugation step, in which 0.68 g/ml of CsCl was used owing to the difference in the density of the two phages. The recovery of phage λ v was 50 to 60%.

Amino acid analysis of λ and λ v ghosts. Protein concentration was determined by a modified biuret method (S. Zamenhof and E. Chargaff, *unpublished data*). An amount of 0.5 to 1 mg of ghost protein prepared by the pyrophosphate method was hydrolyzed in 6 N HCl at 110 C for 24 hr. The amino acid composition of the mixtures was determined by use of a Technicon autoanalyzer. The values are accurate to ±5%.

Thin-layer gel filtration. Sephadex G-150 (superfine) was suspended in a buffer containing 0.1 M citric acid and 8 M urea (*p*H 2.5). The gel was then spread on 20 × 20 cm glass plates and was used for descending chromatography in the same buffer. Samples containing 10 to 20 μg of protein were applied in a volume of 2 to 5 μliters. When the excluded marker substance (Blue dextran 2000) reached the bottom of the plate, the proteins were absorbed onto a superimposed sheet of Whatman 3 MM. The paper was dried and stained with a saturated solution of amido black 10B in methanol containing 10% acetic acid.

Thin-layer electrophoresis. Thin-layer plates of Sephadex G-150 prepared as described above were used as supporting medium for electrophoresis at 400 v by use of a Heathkit model 1 P-32 Regulated Power Supply.

CsCl density gradient separation of phages λ and λ v. Phages λ and λv were mixed in the Tris buffer containing 0.7 g/ml of CsCl and centrifuged at 37,000 rev/min in SW-39 rotor of the Spinco model L ultracentrifuge for 18 hr. Fractions of 10 drops were collected and assayed for phage titer on *E. coli* W-1485/T1, and the density was determined by use of a Bausch & Lomb refractometer.

RESULTS

Formation of protein ghosts of λ. The head of bacteriophage λ, like those of the T-odd bacteriophages (2, 9), is permeable to cations capable of neutralizing deoxyribonucleic acid (DNA). For this reason, Mg^{++} is normally added to the medium to stabilize phage structure. Removal of divalent cations by prolonged dialysis or treatment with pyrophosphate or ethylenediaminetetraacetic acid (EDTA; both effective chelating agents for Mg^{++}) lead to loss of infectivity and concomitant release of DNA (12). Figure 1 shows the result of treating λ with 0.01 M Na pyrophosphate at *p*H 8.4. Loss of turbidity of the phage suspension and increase in its viscosity followed the same kinetics as the loss of infectious units shown in Fig. 1. Therefore, inactivation of the phage corresponded to extrusion of DNA. For preparative purposes, the reaction was carried out at 44 C and 99.9% yields

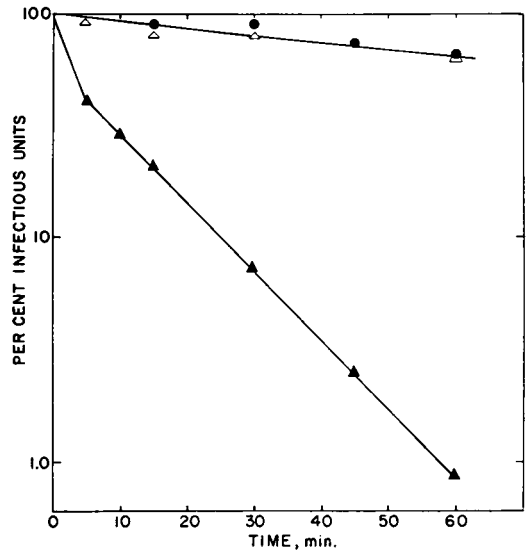

FIG. 1. *Effect of sodium pyrophosphate on phages λ and λ v. Symbols:* ▲, *phage λ in 0.01 M sodium pyrophosphate, pH 8.4, 37 C;* △, *control, λ in 0.01 M sodium phosphate buffer, pH 8.4, 37 C;* ●, *λ v in 0.01 M sodium pyrophosphate, pH 8.4, 37 C. In all experiments, phage suspensions at a concentration of 10^{11}/ml were first dialyzed against 0.01 M NaCl for 1 hr in the cold. An equal volume of warm reagent at a concentration of 0.02 M was added at zero-time and the reaction mixture was maintained at 37 C.*

of ghosts were obtained. Similar results were obtained with EDTA as chelating agent.

The product of the pyrophosphate reaction was primarily "intact ghosts"; i.e., electron micrographs showed empty-headed virus. The ghosts were purified by first dialyzing against a buffer containing 0.067 M Tris, 0.5% NaCl, and 0.001 M $MgSO_4$ (*p*H 7.0) to remove pyrophosphate. Deoxyribonuclease was then added to a concentration of 10 μg/ml, and the suspension was stirred gently at 37 C for 2 hr. The ghosts were sedimented in the SW-39 rotor of the Spinco model L ultracentrifuge at 37,500 rev/min for 1 hr. The ghosts were resuspended in the Tris buffer and resedimented. The pellet was then suspended in 0.1 N ammonium acetate (*p*H 7.0) and passed through a column of Sephadex G-100, equilibrated with the ammonium acetate. The purified ghosts that emerged in the void volume of the column had a ratio of A_{280} to A_{260} of 1.2. In the preparation of large quantities of ghosts, the final Sephadex treatment was replaced by either CsCl density gradient centrifugation or repeated washings in the Tris buffer.

Amino acid analysis. The amino acid analysis of the purified λ ghosts is shown in Table 1 along

TABLE 1. *Amino acid analysis*

Amino acid	λ		λv	
	Molar ratio	No. of residues	Molar ratio	No. of residues
Aspartic acid	.089	18	.094	19
Threonine	.088	18	.089	17
Serine	.074	15	.073	14
Glutamic acid	.102	20	.099	20
Proline	.056	11	.054	11
Glycine	.085	17	.085	17
Alanine	.103	20	.103	20
Valine	.074	15	.075	15
Methionine	.031	6	.029	6
Isoleucine	.031	6	.031	6
Leucine	.061	12	.070	14
Tyrosine	.041	8	.038	8
Phenylalanine	.042	8	.042	8
Lysine	.055	11	.053	11
Histidine	.005	1	.005	1
Arginine	.056	11	.054	11
Cysteine	—	—	—	—
Minimal molecular weight	21,430	197	21,673	198

with the analysis of ghosts of λ v. The amino acid composition shown represents the average of three separate determinations for each phage. Since the variation in amino acid composition within each group of three analyses was as large as the differences shown between λ and λ v, the data can only be taken as an indication that the amino acid compositions are strikingly similar. More detailed studies on the individual proteins of the phage will be necessary to determine whether or not they are identical. It is of interest, however, that neither phage contains any cysteine, eliminating the possibility of disulfide bond formation between subunits.

Effect of purified ghosts on host E. coli. Ghosts of bacteriophage T2 obtained by osmotic shock retain the ability to adsorb to host *E. coli*. These adsorbed ghosts cause a transformation of the bacterial nucleus which is different from the effect of the intact phage but which nonetheless leads to death of the bacterium (3). For comparison, purified ghosts of λ were tested for their ability to kill host *E. coli*. Phage and bacteria were mixed in adsorption medium containing 24.65 mg per liter of $MgSO_4 \cdot 7H_2O$, 5 mg per liter of thiamine, and 20 mg per liter of tryptone, incubated for 30 min at 37 C; the bacteria were plated for viable counts. Over a range of multiplicities from 10 to 200 ghosts per bacterium, no killing effect of λ ghosts was observed. The extent of adsorption of ghosts to cells was not measured directly in these experiments, but the work of Soller, Levine, and Epstein (12) suggests that the tail antigen responsible for adsorption is intact in pyrophosphate ruptured λ.

Formation of protein ghosts of λ v. Since the virulent mutant λ v was not affected by treatment at 37 C with pyrophosphate or EDTA (Fig. 1), it was necessary to devise a method for the efficient production of ghosts of λ v. In the course of studying the effect of a number of reagents, it was observed that the urea, sodium sulfite, cupric sulfate reagent of Dixon and Wardlaw (5) rapidly inactivated λ and λ v. Further experiments identified the Cu^{++} as the effective part of the mixture. Figure 2 shows the effect of 0.01 M $CuCl_2$ in 0.1 N NH_4Cl at *p*H 4.5 on both wild-type λ and the virulent mutant. However, the

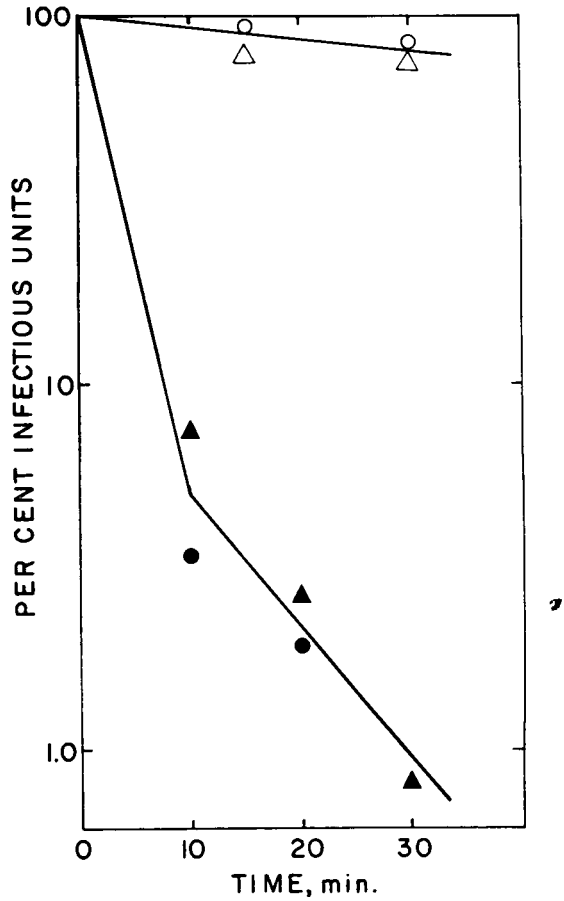

FIG. 2. *Effect of cupric chloride on phages* λ *and* λ v. *Symbols:* ▲, λ *in 0.01 M* $CuCl_2$ *in 0.1 N* NH_4Cl, *pH 4.5, 25 C;* △, *control,* λ *in 0.1 N* NH_4Cl, *pH 4.5, 25 C;* ●, λ v *in 0.01 M* $CuCl_2$ *in 0.1 N* NH_4Cl, *pH 4.5, 25 C;* ○, *control,* λ v *in 0.1 N* NH_4Cl, *pH 4.5, 25 C. Phage suspensions at a titer of* 5×10^{12} *were diluted 100-fold directly into the indicated reagent.*

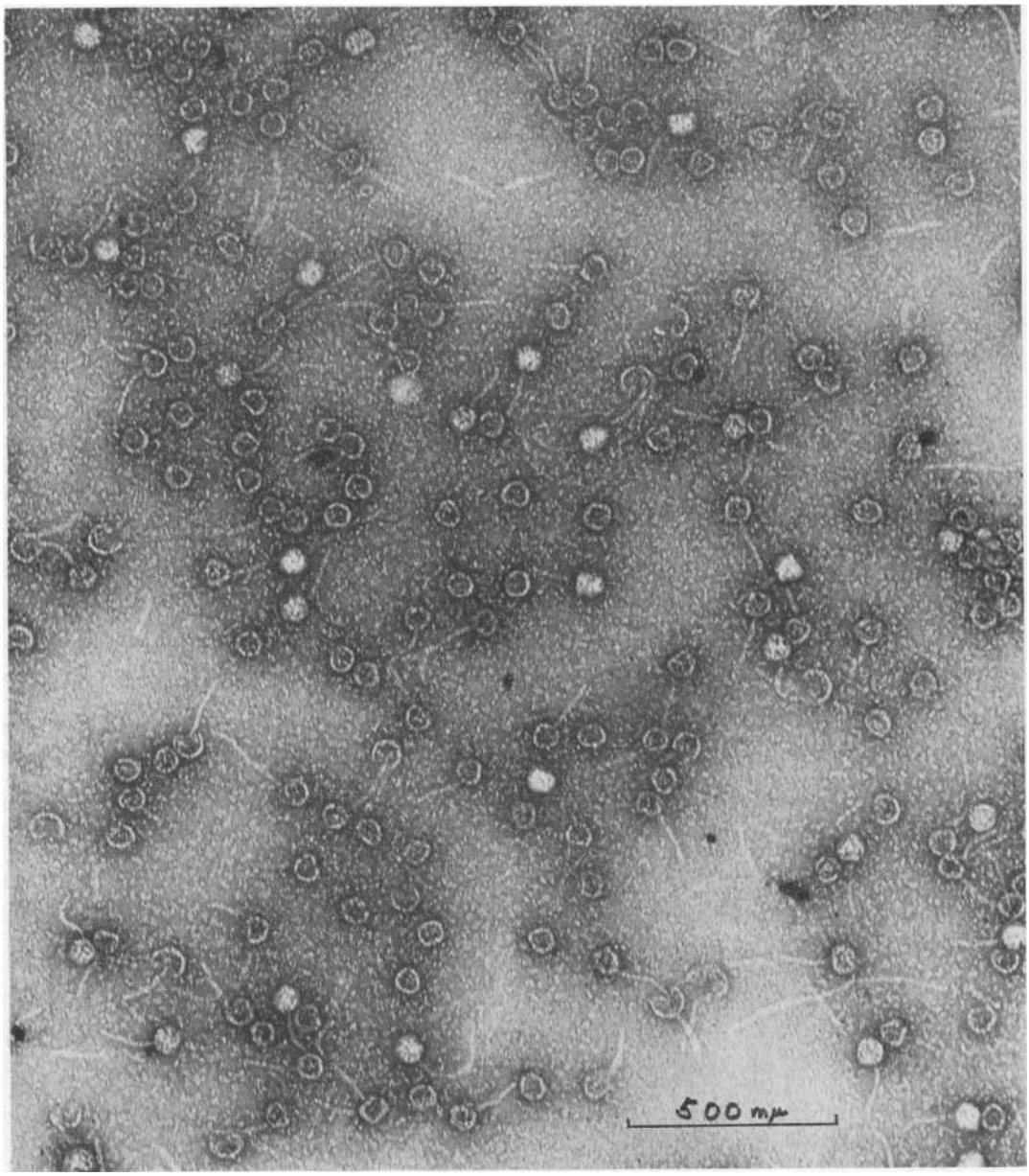

FIG. 3. *Electronmicrograph of the products of the reaction of 0.01 M $CuCl_2$ in 0.1 N NH_4Cl at pH 4.5 with bacteriophage λ. The preparation was negatively stained with phosphotungstic acid and contains phage, intact ghosts, empty heads, and severed tails.*

Cu^{++} reaction was not simply an extrusion of DNA. The inactivation kinetics in Fig. 2 correspond to a gradual precipitation of inactive virus. This was followed much more slowly by liberation of DNA (as measured by increase in viscosity), and a separation of the head and tail of the ghost. The result was a mixture of products: phage, intact ghosts, empty heads, and severed tails. By dialyzing phage against the Cu^{++} reagent, it was possible to reduce the phage titer by a factor of 10^8. The effectiveness of Cu^{++} in the reduction of phage titer was strongly dependent on the other ions making up its coordination complex. Cupric acetate in 0.1 N Tris, for instance, had almost no

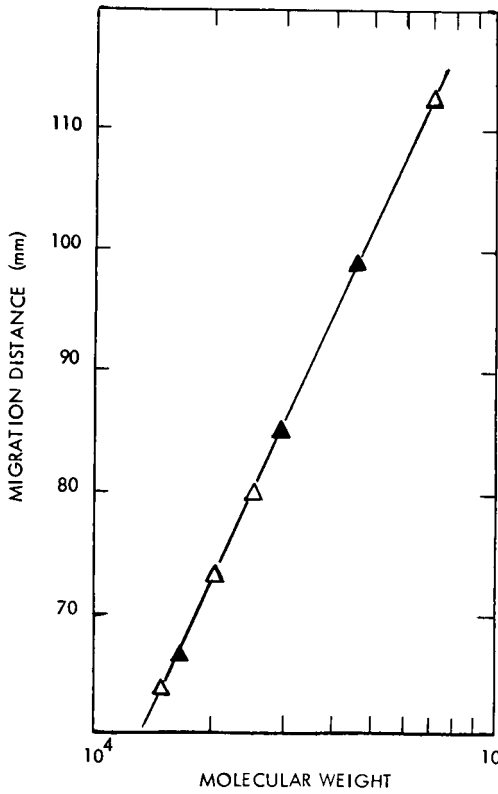

FIG. 4. *Molecular weight estimates by thin-layer gel filtration. The distance migrated by a given protein on Sephadex G-150 in 0.01 M citrate and 8 M urea (pH 2.5) is plotted versus molecular weight. Symbols: △, standard proteins lysozyme, molecular weight 14,400; monomer of β-lactoglobulin, 17,500; chymotrypsinogen, 25,000; and bovine serum albumin, 67,000. ▲, λ ghosts dissolved in 0.01 M citrate and 8 M urea, pH 2.5.*

effect on phage infectivity at a *p*H and concentration comparable to the $CuCl_2$ and NH_4Cl mixture. Cupric acetate in 0.1 N ammonium acetate had an effect that was intermediate between that of cupric acetate in the Tris and that in the NH_4Cl.

The purification of the products obtained from treatment with Cu^{++} reagent began with dialysis against 0.01 M EDTA (*p*H 7.0) in the cold to remove Cu^{++}. Removal of the Cu^{++} led to dispersal of the precipitate, but to no recovery of phage infectivity. After digestion with deoxyribonuclease and sedimentation of the protein products by a purification procedure similar to that used for pyrophosphate-produced ghosts, the components of the mixture could be fractionated by differential centrifugation. Figure 3 shows the products of the Cu^{++} reaction, negatively stained with phosphotungstic acid.

Chemical properties of ghosts. Ghosts of λ and λ v were stable in the *p*H range of 6 to 11.5. Addition of 8 M urea had no effect over this range. Sodium dodecyl sulfate (SDS) at 1% had no disruptive effect at neutral *p*H, but, at alkaline *p*H values, SDS and other surfactants caused a swelling of the ghosts without disrupting the inter-subunit bonds. Treatment with $NaBH_4$ had no effect, as could be expected from the complete absence of cysteine in the phage as shown by amino acid analysis (Table 1).

Inter-subunit linkages were only labilized at *p*H values below 3.0. Pyrophosphate-prepared λ ghosts dissolved very slowly (1 week) in 0.01 M citrate buffer at *p*H 2.5. The rate was increased slightly by lowering the *p*H to 2.0. The rate of solution was greatly accelerated by the addition of 8 M urea to the citrate buffer. Cu^{++} prepared λ v ghosts dissolved only in the urea-citrate mixture.

Subunit structure of λ. The protein components of the solutions of λ v and λ ghosts were separated and identified by two methods: thin-layer gel filtration and thin-layer electrophoresis both with Sephadex as the supporting medium. Both methods separated three component proteins for each phage.

Figure 4 shows molecular weight estimates obtained by thin-layer gel filtration on Sephadex G-150 in citrate-urea buffer (*p*H 2.5). The distance moved on the plate was plotted versus the log of the molecular weight according to the method of Andrews (1). Repeated estimates by this method yield molecular weights which agreed to within ±1,500. Estimates of the relative amounts of the three proteins were obtained from integration of densitometer tracings of the chromatograms and from a crude column separation. These measurements show that the largest protein, with a molecular weight of 47,000, represents a minimum of 55% of the protein of the ghost and is therefore likely to be the subunit of the head. This would correspond to approximately 250 to 300 head subunits per phage. The proteins of molecular weight 30,000 and 16,000 represent approximately 25 and 20% of the protein, respectively. It is not yet possible to assign these two proteins to anatomical regions of the phage.

Figure 5 shows a tracing of a two-dimensional separation of subunits on thin-layer Sephadex G-150. Electrophoresis was run in the horizontal and gel filtration in the vertical, both in citrate-urea buffer (*p*H 2.5). The protein with the greatest positive charge is the one with molecular weight 16,000, followed by the main component at a molecular weight of 47,000. From the relative distances migrated on gel filtration and electrophoresis, it was possible to calculate that the

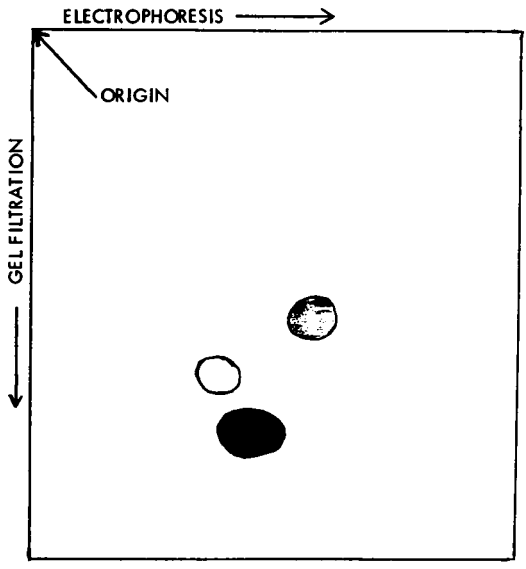

FIG. 5. *Tracing of a two-dimensional separation of a mixture of λ and λ v subunits by gel filtration and electrophoresis. Amounts of 10 μg each of dissolved λ and λ v proteins were mixed and applied to a thin-layer plate of Sephadex G-150. Gel filtration was run as described in Fig. 4. The plate was then rotated by 90° and subjected to electrophoresis at 400 v for 5.5 hr.*

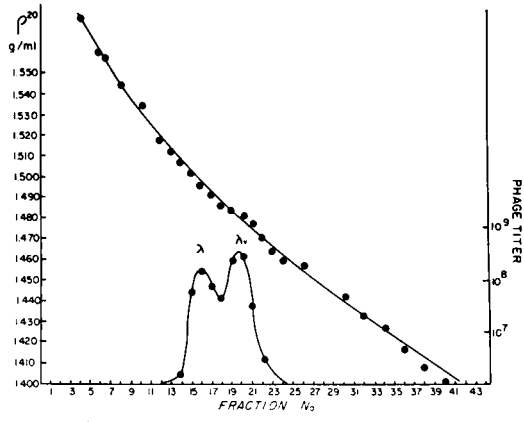

FIG. 6. *Determination of the densities of λ and λ v. Phages λ and λ v were mixed in a buffer containing 0.7 g/ml of CsCl and centrifuged to equilibrium. Fractions of 10 drops were collected and assayed for phage titer, and the density was determined by refractometry.*

net charge on the smallest protein is approximately 1.7 times that of the largest. Because of the high density of positive charge that this represents, it is interesting to speculate on a possible interaction of this protein with DNA.

The two dimensional separation shown in Fig. 5 was done on a mixture of λ and λ v subunits. Within the limits of this method, the proteins of the two related phages are identical. (Sensitivity to small differences in amino acid composition would be increased if electrophoresis could be done closer to neutral *p*H. Unfortunately, the λ proteins are not soluble above *p*H 3.)

Density difference between phage λ and λ v. The determination of the density difference between λ and λ v leads to a quantitative statement about the structure of the virulent mutant. The buoyant density of phage λ v is 0.016 g/ml less than that of λ (Fig. 6). Since the protein components of the two phages appear to be identical, the difference in density of the virulent mutant must be due to a lower DNA content. A calculation of the difference in DNA content by use of the formula of Weigle et al. (15) indicates that λ v contains 16% less DNA than the temperate phage.

Ptashne has reported (13) that the temperate behavior of λ involves the presence of a specific repressor protein. From the study of λ reported here, the deficiency in DNA would be more than sufficient to account for the coding of the repressor molecule and suggests that other genetic information might be required to establish the lysogenic state. However, it is clear from these experiments that the genetic coding for the structural proteins of the virus is unaffected by the mutation to the virulent state.

DISCUSSION

The experiments described above indicate that there are three structural proteins in bacteriophage λ. However, the conditions used for dissolving the ghosts (0.1 M citrate, 8 M urea, *p*H 2.0) are severe, and the possibility arises that the native structure contained only one or perhaps two proteins, which were degraded during preparation. There is considerable evidence, however, to support the view that there are three distinct protein subunits.

First, λ and λ v prepared by entirely different methods, yield the same three proteins. Although the Cu^{++} used in the preparation of λ v is known to catalyze the slow splitting of peptide bonds (11), λ virus prepared by the pyrophosphate method contained the same number of polypeptides. The identity of the three proteins in the two viruses also supports the contention that these proteins are all of viral origin and are not contaminants from the host.

Second, an immunological study (12) has shown that antibodies prepared to a pyrophosphate-ruptured suspension of λ C (a "clear" mutant) react identically with λ C, λ, and a

virulent λ mutant, supporting the conclusion that the structural proteins are unaltered in mutation to the virulent state. This same study showed that at least two distinct antigens, possibly three, could be detected by their individual complement-fixing activity and another by its ability to react with phage neutralizing antibody. This suggests a total of three, and possibly four, antigens in native λ ghosts. If a fourth protein component is present in λ, it must represent less than 5% of the total protein or not be separable by the techniques used in this study.

Third, a consideration of the anatomy of the phage leads to an expectation of at least two, and probably three protein components. From the measurements of Kellenberger (8), Kaiser (7), and Cummings et al. (4), a model for λ can be constructed from which it is possible to calculate the relative amounts of the several structural proteins. This model assumes a spherical head with an outside diameter of 550 A and membrane thickness of 25 A attached to a long cylindrical tail, 1,500 A long and with a 25 A membrane surrounding a 25 A diameter hollow core. Kaiser has also postulated the existence of a core protein (300 A diameter) inside the viral head.

The volume occupied by each of these proteins would be as follows: head protein, 21.6×10^6 A^3; tail, 5.9×10^6 A^3; and core, 14.1×10^6 A^3. The head protein would be present in largest amount (53% by this model in good agreement with the 55% obtained in our experiments). The measured head membrane thickness of 25 ± 9 A is a reasonable dimension for subunits of molecular weight 47,000.

The model predicts that the core protein should represent 34% of the total protein. This exceeds the values of 20% total protein found for the highly positively charged protein of molecular weight 16,000. However, if the core exists, it is not clear that it would be solid as assumed in the model or that it could be obtained in reasonable yield, since only 10% of the ghosts in electronmicrographs show cores (7). On the basis of the Kaiser model, the tail should account for 13% of the protein. The measurements of Cummings et al. (4) of a DNA-free region within the head of λ whose approximate dimensions are 180 by 80 A lead to strikingly different predictions of the relative quantities of the λ proteins. Assuming that the core dimensions refer to an oblate spheroid, the relative volumes occupied by the three proteins would be: head, 70%; core, 5%; and tail, 20%. These models assume that each anatomical region contains only one kind of subunit. Experiments in progress to separate the different anatomical regions and analyze their protein components may prove these models to be an oversimplification.

ACKNOWLEDGMENTS

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